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The severe pathogenicity of alveolar macrophage-depleted ferrets infected with 2009 pandemic H1N1 influenza virus

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ABSTRACT

The *in vivo* role of alveolar macrophages in the infections with 2009 pandemic H1N1 influenza virus is not as yet known. Ferret study shows that alveolar macrophages are critical for lowering the risk of severe outcomes in 2009 pandemic H1N1 influenza virus infections. Up to 40% of the infected ferrets depleted of alveolar macrophages died, with elevated body temperature and major loss of body weight in contrast to infected ferrets not depleted of alveolar macrophages. The higher viral titers in the lungs were detected in infected ferrets depleted of alveolar macrophages than infected ferrets not depleted of alveolar macrophages 5 days after infection. The inflammatory chemokines were induced at greater levels in the lungs of infected ferrets depleted of alveolar macrophages than in those of infected ferrets not depleted of alveolar macrophages. Our study implies that alveolar macrophages are important for controlling the infections of 2009 pandemic H1N1 influenza virus.

Introduction

Influenza virus belongs to Orthomyxoviridae family which include influenza A, B, and C genera (Bouvier and Palese, 2008). The genome of influenza virus is segmented negative-strand RNA that requires RNA-dependent RNA polymerase to replicate. Influenza A virus is further subtyped based on the antigenicity of its surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) and is responsible for human pandemics (Bouvier and Palese, 2008). Influenza A virus has a broad spectrum of host ranges including humans and animals such as birds, pigs, horses, seals, mink, and whales (Webster et al., 1992). Most known subtypes of influenza A viruses including 16 HA and 9 NA subtypes are circulating in aquatic birds (Fouchier et al., 2005; Webster et al., 1992).

Influenza A viruses are responsible for human pandemics. During the 20th century, the devastating pandemic that claimed over 50 million human lives was caused by avian-like H1N1 influenza virus in 1918 (Johnson and Mueller, 2002; Taubenberger et al., 1997; Taubenberger et al., 2005), and the pandemics caused by the reassorted viruses between genes of avian and human influenza viruses occurred in 1957 and 1968 (Kawaoka et al., 1989; Scholtissek et al., 1978), The 1957 and 1968 pandemics were caused by H2N2 and H3N2 influenza viruses. During the 21st century, we experienced a pandemic caused by a swine-origin H1N1 influenza virus in 2009. In April, 2009 a swine-origin H1N1 influenza virus that was antigenically distinct from the seasonal H1N1 influenza virus was found in humans (CDC, 2009). After that, the novel H1N1 influenza virus spread worldwide, resulting in the declaration of a global pandemic by the World Health Organization on 11 June, 2009. The clinical signs of most people infected with this virus were mild, but young people and the immune-compromised people with conditions such as asthma, diabetes, severe obesity, lung problem and pregnancy suffered from severe clinical signs including death (Jain et al., 2009). The genetic analysis of this virus shows that it consists of a unique combination of genes from viruses that circulated in pigs in North America, Europe, and Eurasia (Garten et al., 2009). The NA and matrix (M) genes originated from viruses that belong to the Eurasian swine genetic lineage, the HA, nucleoprotein (NP), and nonstructural (NS) genes are derived from viruses of classical swine lineage, and polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acidic (PA) genes are from the viruses of swine triple reassortant lineage (Garten et al., 2009).

Macrophages are involved in maintaining the homeostasis of organs, rendering host defense, and wound healing (Duan et al., 2012; Murray and Wynn, 2011). Macrophages are functionally plastic,

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undergoing polarization into unique phenotypes relying on the external stimuli (Gordon and Martinez, 2010). The classically activated macrophages produce the inflammatory cytokines and chemokines and are involved in killing pathogens, while the alternatively activated macrophages possess immunoregulatory properties, inducing wound healing and modifying the extracellular matrix by secretion of proteases and growth factors (Gordon and Martinez, 2010). Macrophage precursors generated from the committed hematopoietic stem cells in the bone marrow are released into the circulation as a form of monocytes and are seeded into tissues throughout the body, becoming macrophages or dendritic cells (Murray and Wynn, 2011). Alveolar macrophages reside in the alveolar airspaces, and over 98% of the cells in the alveolar airspaces are alveolar macrophages (9). In the lungs, they live in a quiescent and immunosuppressive state that can exert pathogen phagocytosis and T cell trafficking in the early response of viral and bacterial infection (33). The *in vitro* or *in vivo* studies imply that macrophages are involved in pathogenesis and immune responses in influenza virus infections (Gao et al., 2012; Kim et al., 2008; Osterlund et al., 2010; Tate et al., 2010; Tumpey et al., 2005). The 2009 pandemic H1N1 influenza virus triggered the weak induction of cytokines in human macrophages and dendritic cells (Osterlund et al., 2010), and the infection of swine macrophageswith the 2009 pandemic H1N1 influenza virus strongly induced proinflammatory cytokines such as interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α (Gao et al., 2012). An *in vivo* depletion study of alveolar macrophages in mice showed that alveolar macrophages are important for controlling the infections with 1918 pandemic H1N1 or seasonal H3N2 influenza viruses (Tate et al., 2010; Tumpey et al., 2005). The depletion of alveolar macrophages in a pig, a natural host of influenza virus caused more severe disease than non-depleted infected pigs with a seasonal H1N1 influenza virus (Kim et al., 2008).

Ferrets (*Mustela putorius* furo) have been used for studying the pathogenesis caused by influenza viruses since they can show clinical signs similar to humans infected with influenza viruses (Belser et al., 2011a, 2011b; Kang et al., 2011; Kim et al., 2009; Munster et al., 2009; Roberts et al., 2012; Seibert et al., 2010). In ferrets infected with 2009 pandemic H1N1 influenza virus the higher inflammatory cytokines including IL-6, interferon (IFN)-α, and TNF-α were induced than in ferrets infected with seasonal H1N1 influenza virus (Kang et al., 2011). Ferrets infected with 2009 pandemic H1N1 influenza virus suffered from more severe disease than those infected with seasonal H1N1 influenza virus (Munster et al., 2009).

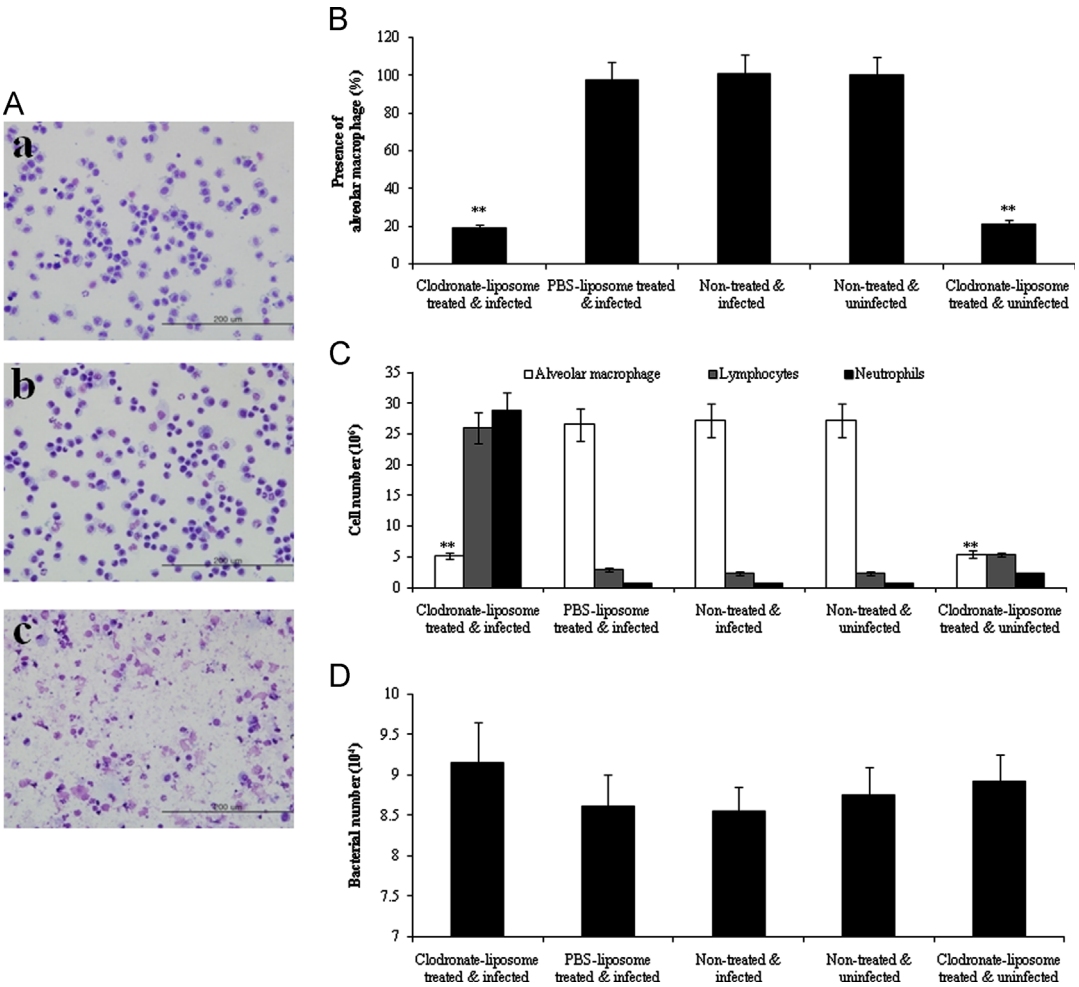

Fig. 1. The depletion efficiency of alveolar macrophages and the infiltration of cells and bacteria in the broncho-alveolar space in the infected ferrets. Ferrets ($n=3$ per group) were depleted of alveolar macrophages by treatment with clodronate-liposome for 7 days before they were infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009). The BAL samples were treated with Wright staining to determine the depletion efficiency. After ferrets were infected with 2009 pandemic H1N1 influenza virus, the BAL samples collected from ferrets were stained with Wright reagent to enumerate the infiltration cells in the lungs of ferrets or smeared on agar plate to determine bacterial infections. Statistical analysis was performed between data of ferrets treated with clodronate-liposome and PBS-liposome. $^{**}P < 0.001$. (A) The cell pictures (× 200) in BAL samples from ferrets before infections a, cells from the untreated and uninfected ferrets; b, cells from the uninfected ferrets treated with PBS-liposome; c, cells from the uninfected ferrets treated with clodronate-liposome. (B) The depletion efficiency of alveolar macrophages in ferrets before infection. (C) The infiltrating cells in the broncho-alveolar space of infected ferrets. (D) The infiltration bacteria in the broncho-alveolar space of infected ferrets.

The current study investigated the role of alveolar macrophages in ferrets infected with 2009 pandemic H1N1 influenza virus. We studied the clinical signs including mortality, the change of body temperature, and body weights in ferrets depleted of alveolar macrophages with clodronate-liposome and infected with 2009 pandemic H1N1 influenza virus. We also determined the induction of cytokines and chemokines in the lungs of ferrets infected with 2009 pandemic H1N1 influenza virus to explain the underlying mechanism of disease severity of infected ferrets depleted of alveolar macrophages.

Results

Clodronate-liposome efficiently depletes alveolar macrophages in the lungs of ferrets

We depleted the alveolar macrophages in ferrets using the clodronate-liposome that causes apoptosis of macrophages before ferrets were infected with 2009 pandemic H1N1 influenza virus. We determined the depletion efficiency of alveolar macrophages in ferrets with clodronate-liposome (Fig. 1A and B). When the cells collected from the lungs of ferrets treated with clodronate-liposome for 7 days were stained with Wright staining solution the cells from untreated or PBS-liposome treated ferrets contained many intact alveolar macrophages (Fig. 1A-a and A-b), while the cells from clodronate-liposome treated ferrets contained small number of alveolar macrophages with much cell-disrupted debris (Fig. 1A-c). We calculated the depletion efficiency of alveolar macrophages in ferrets with clodronate-liposome by counting the cell numbers compared to the number of alveolar macrophages from untreated ferrets (Fig. 1B). The treatment of ferrets with clodronate-liposome resulted in about 81% depletion of alveolar macrophages, while no depletion of alveolar macrophages occurred in the infected ferrets treated with PBS-liposome, in the non-treated infected ferrets, and in the non-treated uninfected ferrets (Fig. 1B). To find out the possible recruitment of cells such as alveolar macrophages, lymphocytes, and neutrophils in the lungs of ferrets infected with 2009 pandemic H1N1 influenza virus, we enumerated the total cell number in the BAL samples of infected ferrets at 5 days after infection (Fig. 1C). The number of alveolar macrophages in the infected ferrets treated with clodronate-liposome remained lower than that of control groups including PBS-liposome treated infected ferrets, non-treated infected ferrets, and non-treated uninfected ferrets (Fig. 1C), whereas the number of lymphocytes and neutrophils was higher in the infected ferrets treated with clodronate-liposome than that in control groups such as PBS-liposome treated infected ferrets, non-treated infected ferrets, and non-treated uninfected ferrets (Fig. 1C). Lymphocyte and neutrophil counts in clodronate-liposome treated ferrets were statistically different from those in PBS-liposome treated ferrets (Fig. 1C). We also measured the total

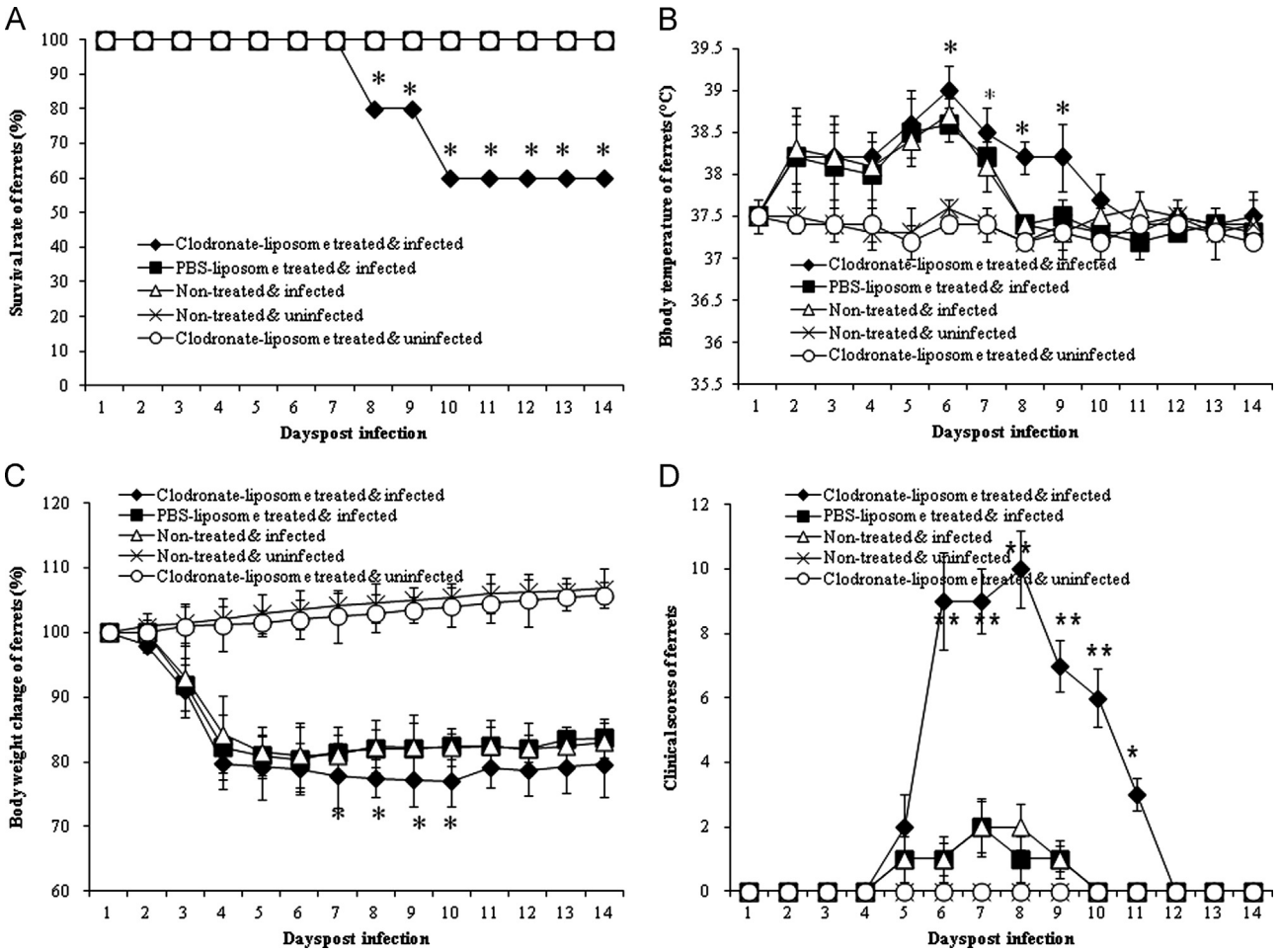

Fig. 2. The mortality, the change of body temperatures, and body weights, and clinical scores of infected ferrets. Ferrets ($n=5$ per group) treated with clodronate-liposome or PBS-liposome were i.n. infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009) and were monitored for deaths, the change of body weight, the change of rectal body temperatures for 14 days (once per day) after infections. The clinical scores of infected ferrets were calculated as described in materials and methods. The statistical analysis was performed between data of infected ferrets treated with clodronate-liposome and PBS-liposome. $^{*}P<0.05$; $^{**}P<0.001$. (A) The survival rate of infected ferrets. (B) The change of body temperatures of infected ferrets. (C) The change of body weights of infected ferrets. (D) The clinical scores of infected ferrets.

bacterial number in BAL samples collected from ferrets at 5 days after infection to find out whether the depletion of alveolar macrophages could affect the bacterial infection in the lungs of ferrets (Fig. 1D). The number of bacteria (9.15×10^4) in the lungs of infected ferrets treated with clodronate-liposome was slightly higher than that of bacteria in the lungs of control groups including PBS-liposome treated infected ferrets (8.6×10^4), non-treated infected ferrets (8.5×10^4), and non-treated uninfected ferrets (8.7×10^4) (Fig. 1D). Bacterial number in clodronate-liposome treated ferrets was not statistically different from those in PBS-liposome treated ferrets (Fig. 1D).

Alveolar macrophage-deficient infected ferrets showed more severe clinical signs than alveolar macrophage-intact infected ferrets

Ferrets treated with clodronate-liposome were infected with 2009 pandemic H1N1 influenza virus and were monitored for survival rate, the change of body temperature, the change of body weight, and clinical score (Fig. 2A–D). When we evaluated the survival rate the alveolar macrophage-deficient infected ferrets showed 60% of survival rate, while control groups such as PBS-liposome treated infected ferrets, non-treated infected ferrets, and non-treated uninfected ferrets showed 100% of survival rate (Fig. 2A). Survival rate in clodronate-liposome treated ferrets was statistically different from those in PBS-liposome treated ferrets (Fig. 2A). When we measured the rectal temperature the infected ferrets treated with clodronate-liposome showed higher body temperature than control groups. The peak temperature of infected ferrets treated with clodronate-liposome was 39 °C at 6 days after infection, and that of infected ferrets treated with PBS-liposome was 38.6 °C at 6 days after infection (Fig. 2B). Body temperature of non-treated infected ferrets was 38.7 °C at 6 days after infection, and that of non-treated uninfected ferrets was 37.6 °C at 6 days after infection (Fig. 2B). Body temperature in clodronate-liposome treated ferrets was statistically different from those in PBS-liposome treated ferrets (Fig. 2B). The infected ferrets treated with clodronate-liposome suffered from more marked weight loss than those treated with PBS-liposome and untreated infected ferrets (Fig. 2C). The mean body weight of infected ferrets treated with clodronate-liposome was about 77% of original weight at 10 days after infection, but that of infected ferrets treated with PBS-liposome was 82.5% of original weight at 10 days after infection (Fig. 2C). The mean body weight of non-treated infected ferrets was 82.3% at 10 days after infection, and that of non-treated uninfected ferrets was 105.5% at 10 days after infection (Fig. 2C). Body-weight loss in clodronate-liposome treated ferrets was statistically different from those in PBS-liposome treated ferrets (Fig. 2A). The clinical scores were recorded based on activity and respiratory clinical signs of infected ferrets (Fig. 2D). The infected ferrets treated with clodronate-liposome showed higher clinical scores than those treated with control groups including PBS-liposome treated and infected ferrets. The sum of clinical scores of infected ferrets treated with clodronate-liposome and PBS-liposome was 10 and 1 at 8 days after infection, respectively (Fig. 2D). The sum of clinical scores of non-treated infected ferrets and non-treated uninfected ferrets was 2 and 0 at 8 days after infection (Fig. 2D). When we studied viral titers in the lungs of ferrets the lungs of infected ferrets treated with clodronate-liposome contained higher viral titers than those of infected ferrets treated with PBS-liposome or untreated at 5 days after infection. The mean viral titer in the lungs of infected ferrets treated with clodronate-liposome was 6.75 EID_{50}/ml, whereas that of infected ferrets treated with PBS-liposome was 4.50 EID_{50}/ml (Fig. 3). The mean viral titer in the lungs of non-treated infected ferrets was 4.75 EID_{50}/ml, and that in the lungs of non-treated uninfected ferrets was less than 1.0 EID_{50}/ml (Fig. 3).

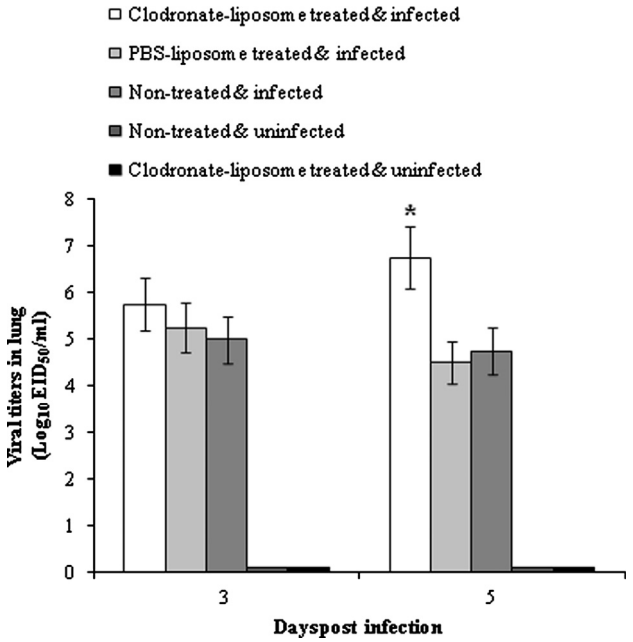

Fig. 3. The viral titers in the left cranial lung lobe of infected ferrets. Ferrets ($n=3$ per group) treated with clodronate-liposome or PBS-liposome were i.n. infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009) and were euthanized at 3 or 5 days after infection to collect the left cranial lobes of lungs. The collected lung tissues were homogenized in PBS (pH 7.4) before the viral titers were determined by log 10 EID_{50}/ml. The statistical analysis was performed between viral titers of infected ferrets treated with clodronate-liposome and PBS-liposome. $^*P < 0.05$.

Alveolar macrophage-deficient infected ferrets showed greater pathological damage to the lung than alveolar macrophage-intact infected ferrets

We compared the pathological damage to the lungs of infected ferrets treated with clodronate-liposome with that of control groups including the infected ferrets treated with PBS-liposome. The gross legion of lungs of infected ferrets treated with clodronate-liposome showed pulmonary consolidation on the dorsal right caudal area and the dorsal right caudal area at 3 days after infection (Fig. 4A e-1 and e-2) and the pulmonary consolidation on the dorsal left cranial and ventral left cranial area and dorsal left cranial and ventral left cranial area at 5 days after infection (Fig. 4A f-1 and f-2), while that of lungs of infected ferrets treated with PBS-liposome showed mild pneumonia compared to those of infected ferrets treated with clodronate-liposome (Fig. 4A c-1, c-2, d-1, and d-2). The gross lesion of lungs of non-treated infected ferrets was similar to that of infected ferrets treated PBS-liposome (Fig. 4A g-1, g-2, h-1, and h-2), and that of lungs of non-treated uninfected ferrets not showing any pneumonia (Fig. 4A a-1, a-2, b-1, and b-2).

The gross lesion percentage of lungs showing pneumonia was also higher in infected ferrets treated with clodronate-liposome than that in infected ferrets treated with PBS-liposome. The percentage showing pneumonia lesion of lungs of infected ferrets treated with clodronate-liposome was 30 at 5 days after infection, while that of lungs infected ferrets treated with PBS-liposome was 12 at 5 days after infection (Fig. 4B). The percentage showing pneumonia lesion of lungs of non-treated infected ferrets was 15 at 5 days after infection and that of lungs non-treated uninfected ferrets was 0 at 5 days after infection (Fig. 4B).

The staining of lung tissues with H&E also showed that the lung tissues of infected ferrets treated with clodronate-liposome contained more severe damage compared to those of infected ferrets treated with PBS-liposome. The infiltrated inflammatory cells were

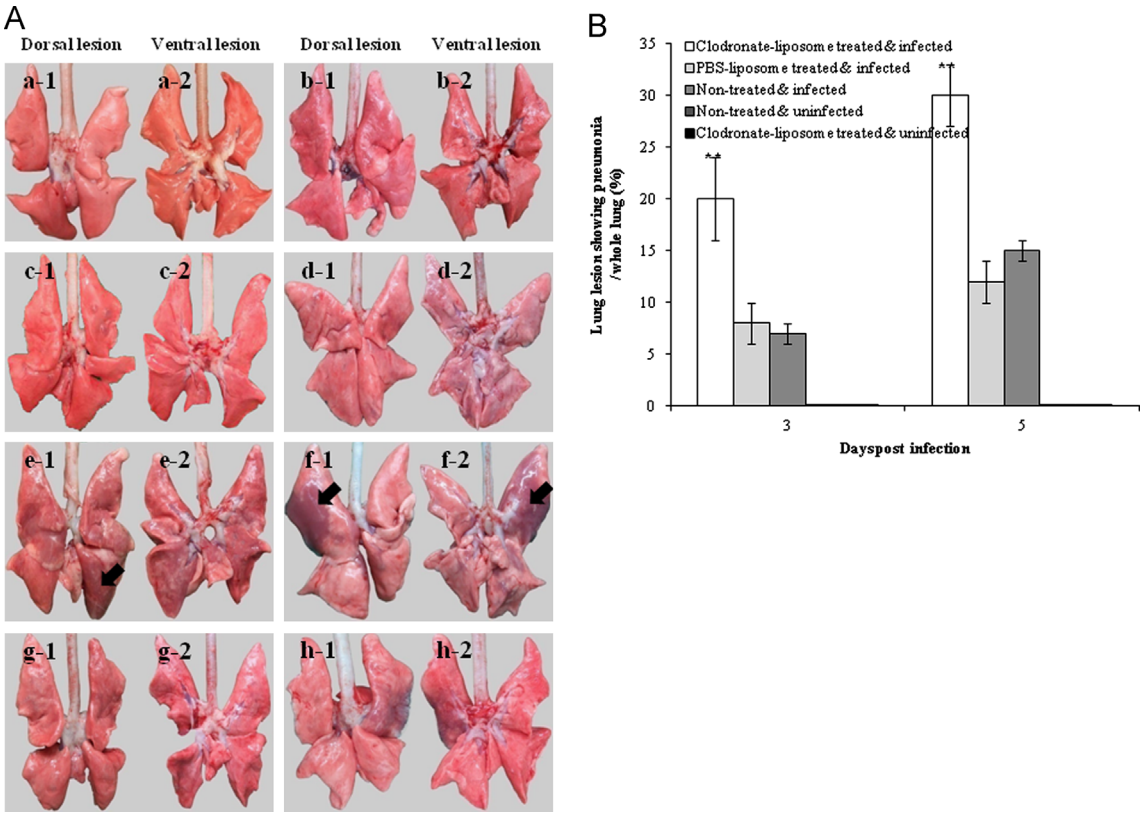

Fig. 4. The gross lesion and pneumonia percentage of lungs of infected ferrets treated with clodronate-liposome. Ferrets ($n=3$ per group) treated with chlodronate-liposome or PBS-liposome were i.n. infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009) and were euthanized at 3 or 5 days to collect lungs. We showed one ferret's lung per group since the others are similar in the group. The gross appearance of lungs was taken a picture (A). (a-1 and a-2) The lungs of untreated and uninfected ferrets. (b-1 and b-2) The lungs of clodronate-liposome treated and uninfected ferrets. (c-1 and c-2) The lungs of PBS-liposome treated and infected ferrets at 3 days after infection. (d-1 and d-2) the lungs of PBS-liposome treated and infected ferrets at 5 days after infection. (e-1 and e-2) The lungs of clodroante-liposome treated and infected ferrets at 3 days after infection. (f-1 and f-2) the lungs of clodroante-liposome treated and infected ferrets at 5 days after infection. (g-1 and g-2) The lungs of untreated and infected ferrets at 3 days after infection. (h-1 and h-2) The lungs of untreated and infected ferrets at 5 days after infection. (B) The surface-area percentage showing pneumonia lesion out of whole lungs of ferrets ($n=3$ per group) on 3 and 5 days after infection was measured. The statistical analysis was performed between percentage of lungs showing pneumonia of infected ferrets treated with clodronate-liposome and PBS-liposome. $^{**}P<0.001$.

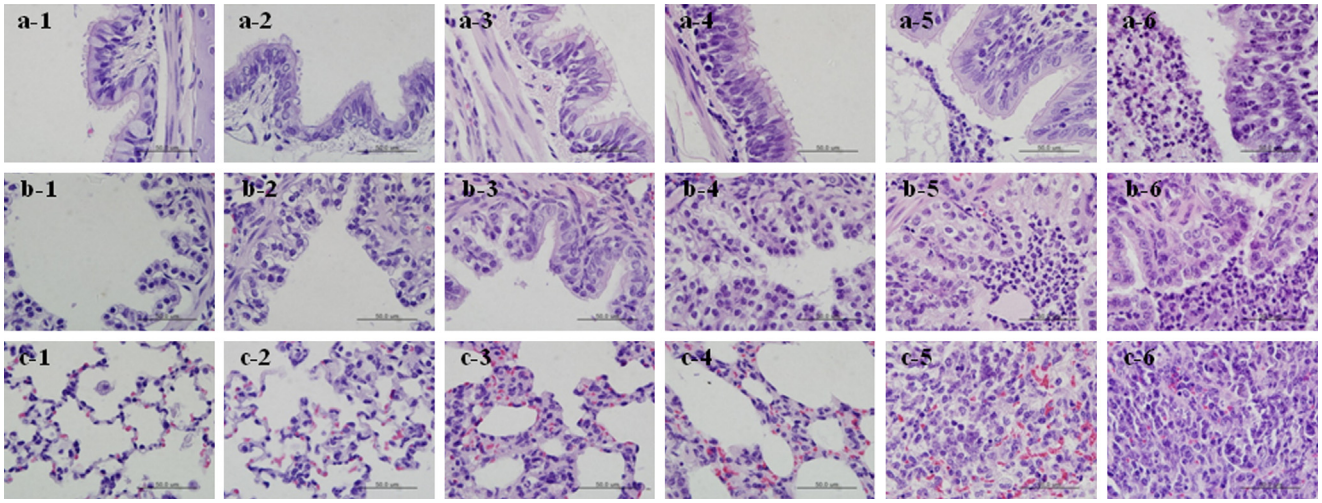

Fig. 5. The histopathological change in the left cranial lung lobe of infected ferrets. Ferrets ($n=3$ per group) treated with clodronate-liposome or PBS-liposome were i.n. infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009) and were euthanized at 3 or 5 days to collect lungs. The collected lungs were stained with H&E and were taken a picture ($\times$ 400). (a-1) Bronchus of untreated and uninfected ferret. (a-2) Bronchus of clodronate-liposome treated and uninfected ferret. (a-3) Bronchus of PBS-liposome treated and infected ferrets at 3 days after infection. (a-4) Bronchus of PBS-liposome treated and infected ferrets at 5 days after infection.

more in the bronchial tissues of infected ferrets treated with clodronate-liposome (Fig. 5a-5 and a-6) than in those of infected with treated with PBS-liposome (Fig. 5a3 and a-4). The lumen of bronchioles of infected ferrets treated with clodronate-liposome (Fig. 5b-5 and b-6) contained more mucus and inflammatory cells than that of bronchioles of infected ferrets treated with PBS-liposome (Fig. 5b-3 and b-4). The alveolar space of lungs of ferrets treated with clodronate-liposome was filled with lots of infiltrated inflammatory cells such lymphocytes and macrophages (Fig. 5c-5 and c-6), but that of lungs of ferrets treated with PBS-liposome was infiltrated with few

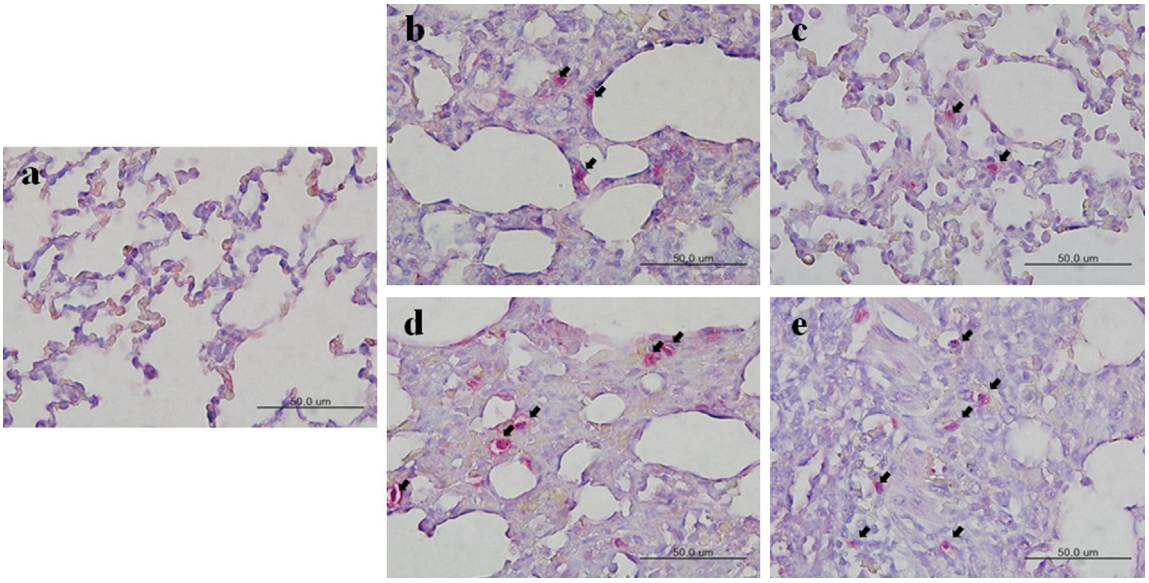

Fig. 6. The antigen staining in the left cranial lung lobe of infected ferrets. The alveolar tissues from ferrets of Fig. 5 were stained with mouse influenza A virus anti-nucleoprotein antibody, biotin-labeled goat anti-mouse immunoglobulin, VECTASTAIN ABC-AP, and Vector red alkaline phosphatase substrate (Vector, USA). We did not show the pictures of bronchi and bronchioles of ferrets since no antigen was detected in these tissues. Arrow: positive staining. (a) Alveolar tissue of untreated and uninfected ferrets. (b) Alveolar tissue of PBS-liposome treated and infected ferrets at 3 days after infection. (c) Alveolar tissue of PBS-liposome treated and infected ferrets at 5 days after infection. (d) Alveolar tissue of clodronate-liposome treated and infected ferrets at 3 days after infection. (e) Alveolar tissue of clodronate-liposome treated and infected ferrets at 5 days after infection.

number of inflammatory cells (Fig. 5c-3 and c-4). Bronchi, bronchioles, and alveoli of uninfected ferrets did not show the accumulation of inflammatory cells (Fig. 5a-1 a-2, b-1, b-2, c-1, and c-2).

When the viral antigens were detected with influenza NP specific antibody the alveolar cells of lungs of infected ferrets treated with clodronate-liposome (Fig. 6d and e) contained higher number of positively stained cells than those of infected ferrets treated with PBS-liposome (Fig. 6b and c). Pneumocytes were dominantly infected (Fig. 6d and e). Alveolar cells of non-treated uninfected ferret were not stained (Fig. 6a). Antigens were not detected in bronchi and bronchioles (data not shown).

Chemokines, not cytokines were induced higher in the lungs of alveolar macrophage-deficient infected ferrets than in those of alveolar macrophage-intact infected ferrets

It has been suggested that the inflammatory responses may contribute to pathogenicity caused by influenza virus infection. We quantified the mRNA of cytokines and chemokines induced in the lungs of ferrets. The cytokine induced were similar in the lungs between infected ferrets treated with clodronate-liposome and infected ferrets treated with PBS-liposome with the exception of IL-6 and IFN-γ (Fig. 7A), but the chemokines were induced higher in the lungs of ferrets treated with clodronate-liposome than in those of ferrets treated with PBS-liposome (Fig. 7B). Cytokine s, IL-6 (1.9-fold increase on 5 days p.i.) and IFN-γ(1.9-fold increase on 3 days p.i.) were induced more in the lungs of infected ferrets treated with clodronate-liposome than in those of infected ferrets treated with PBS-liposome (Fig. 7A). Chemokines, CCL8 that is chemotactic for and activates many different immune cells including mast cells, eosinophils and basophils, CCL11 that selectively recruits eosinophils, CXCL9 that attracts T lymphocytes, CXCL10 that is chemotactic for monocytes, macrophages, T cells, NK cells, and dendritic cells, and CXCL11 that is chemotactic for activated T lymphocytes, were induced higher in the lungs of ferrets treated with clodronate-liposome than in those of ferrets treated with PBS-liposome. The induction of cytokines and chemokines in the lungs of clodronate-liposome treated infected ferrets was similar to that in the lungs of non-treated infected ferrets (Fig. 7A and B).

Discussion

Swine-origin pandemic H1N1 influenza occurred in 2009 and caused severe disease in humans. The information on the *in vivo* role of alveolar macrophages that constitutively reside in alveolar space of lungs of humans and animals against 2009 pandemic H1N1 influenza virus is limited. We studied the role of alveolar macrophages in ferrets by treatment with clodronate-liposome to trigger apoptosis of alveolar macrophages. Ferrets depleted of alveolar macrophages and infected with 2009 pandemic H1N1 influenza virus suffered from more severe clinical signs than those containing intact alveolar macrophages.

We showed that the depletion of alveolar macrophages in ferrets by clodronate-liposome before infection with 2009 pandemic H1N1 influenza virus resulted in up to 40% mortality, while no mortality of control infected ferrets whose alveolar macrophages were not depleted was observed. In addition, the higher body temperatures, greater reduction of body weights, and higher viral titers in the lungs of infected ferrets depleted of alveolar macrophages were detected than in those of infected intact ferrets. The previous studies of the *in vivo* role of alveolar macrophages using influenza-infected mouse and pig models similarly showed that the depletion of alveolar macrophages using clodronate-liposome could lead to higher mortality and more increased clinical signs including reduced body weight than influenza-infected animals that were not depleted of alveolar macrophages (Kim et al., 2008; Tate et al., 2010; Tumpey et al., 2005). When mice depleted of alveolar macrophages by clodronate-liposome were infected with 1918 HA/NA:Tx/91 influenza virus the uncontrolled virus growth and the higher mortality were observed (Tumpey et al., 2005). Another study showed that different pathogenicity was observed in mice depleted of alveolar macrophages and infected with two strains of influenza viruses, A/PR/8/34 (H1N1) and BJx109 (H3N2) (Tate et al., 2010). Depletion of alveolar macrophages in the air ways by clodronate-liposome resulted in the severe pneumonia in

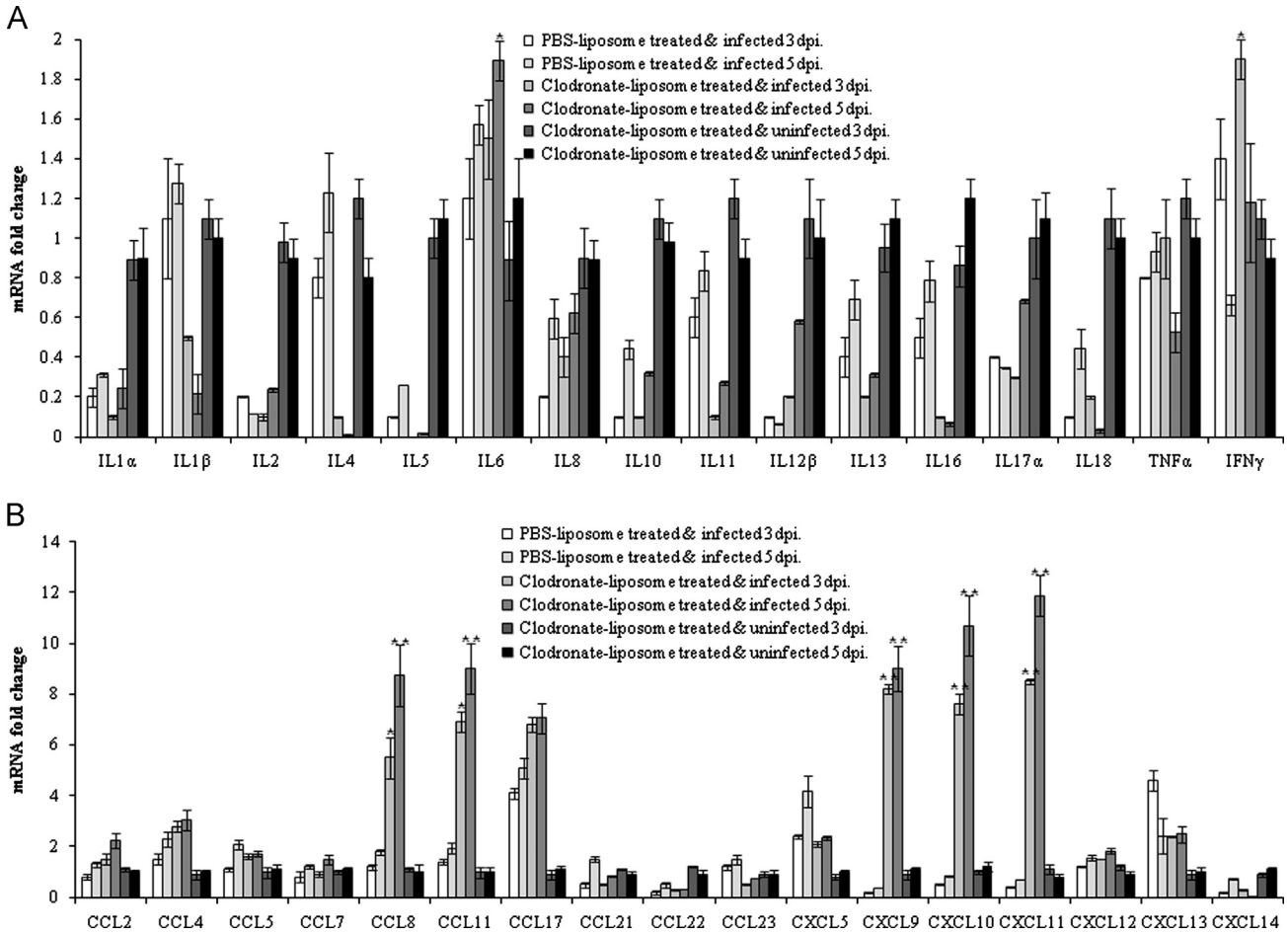

Fig. 7. Quantification of cytokine and chemokine mRNA induced in the left cranial lung lobe of infected ferrets treated with clodronate-liposome. Ferrets ($n=3$ per group) treated with chlodronate-liposome or PBS-liposome were i.n. infected with 2009 pandemic H1N1 influenza virus (A/California/07/2009) and were euthanized at 3 or 5 days to collect lungs. The induced mRNA was quantified by real-time PCR using primers specific for ferret's cytokines and chemokines. Statistical analysis was performed between data of ferrets treated with clodronate-liposome and PBS-liposome. $^{*}P<0.05$, $^{**}P<0.001$. (A) Cytokines; (B) Chemokines.

BJx109-infected mice, while the depletion of alveolar macrophages did not affect disease severity in A/PR/8/34 (H1N1)-infected mice. Our previous study using an influenza virus-natural host, a pig, suggested that alveolar macrophages are critical for controlling a seasonal influenza virus (H1N1). The infected pigs depleted of alveolar macrophages by clodronate-liposome had 40% mortality (Kim et al., 2008). The severe pathogenicity of clodronate-liposome treated ferrets which were infected with 2009 pandemic H1N1 influenza virus may be also partially due to the depletion of dendritic cells (DCs) since mice which were i.t. treated with clodronate-liposome resulted in depletion of alveolar macrophages and DCs (Bosio and Dow, 2005; McGill et al., 2008).

Meanwhile, the previous studies on pathogenicity of 2009 pandemic H1N1 influenza virus using ferrets not depleted of alveolar macrophages showed that the infected ferrets did not die even though this virus caused more severe disease than seasonal H1N1 influenza virus (Itoh et al., 2009; Kang et al., 2011; Munster et al., 2009). Ferrets infected with 2009 pandemic H1N1 influenza virus displayed higher body temperatures, greater reduction in body weight, and higher viral titers in trachea and lungs than those infected with seasonal H1N1 influenza virus (Kang et al., 2011). Ferrets infected with 2009 pandemic H1N1 influenza virus showed more extensive virus replication in the respiratory tracts than a seasonal H1N1 influenza virus. The replication of 2009 pandemic H1N1 influenza virus occurred in the tracheae, bronchi, and bronchioles, but that of seasonal H1N1 influenza virus was limited to the nasal cavity of ferrets (Munster et al., 2009). The pathogenesis study using the pregnant mice also showed that 2009 pandemic H1N1 influenza virus could cause more severe diseases than seasonal H1N1 influenza virus (Kim et al., 2012).

We showed that the viral titers in the lungs of clodronate-liposome treated ferrets was higher than those in the lungs of PBS-liposome treated ferret on 5 days p.i., but that no difference in the viral titers in the lungs of ferrets was observed on 3 days p.i. The similar viral titers in the lungs of ferrets on 3 days p.i. suggest that alveolar macrophages may not be involved in inhibiting influenza virus infection in the lungs of ferrets.

Our results showed that the number of bacteria in the lungs of infected ferrets treated with clodronate-liposome was slightly higher than that of bacteria in the lungs of control groups including PBS-liposome treated and infected ferrets without statistical significance. Bacterial species was not identified in this study since no significant difference was observed in the both groups of ferrets. The previous study on the cystic fibrosis reported that bacteria such as *Staphylococcus*, *Bacillus*, *Enterococcus* and *Streptococcus* species were dominantly detected in BAL samples in the ferrets (Sun et al., 2010).

Our study showed that chemokines such as CCL8, CCL11, CCL17, CXCL19, CXCL10, and CXCL11 involved in recruiting the inflammatory cells were induced higher in the lungs of infected ferrets treated with clodronate-liposome than in those of infected ferrets treated with PBS-liposome. The infiltration of many inflammatory cells in the lungs of infected ferrets treated with clodronate-liposome

than in those of infected ferrets treated with PBS-liposome may be influenced by the increased induction of chemokine genes. The previous studies showed that the inflammatory responses could contribute to the pathogenicity of influenza viruses (Baskin et al., 2009; Cameron et al., 2008). Macaques infected with lethal H5N1 influenza virus suffered from the severe disease and showed the marked elevation of genes related to interferons, inflammatory, and innate immunity in the lungs (Baskin et al., 2009). The lungs of ferrets infected with lethal H5N1 influenza virus were also elevated with genes related to interferon responses and chemokines (Cameron et al., 2008).

In conclusion, our study showed that alveolar macrophages are important in defending a ferret, an important animal model for human influenza from the infections by 2009 pandemic H1N1 influenza virus.

Materials and methods

Virus

Swine-origin 2009 pandemic H1N1 influenza virus (A/California/07/2009) was kindly provided by World Health Organization (WHO)-collaborating center CDC (USA Centers for Disease Control and Prevention) and propagated in the amniotic cavities of 10-day-old hens' eggs prior to being used for the study.

Animals

About 7-to-8-week-old ferrets obtained from Path valley farm (Spring Run, PA, USA) were serologically negative to the human influenza viruses, 2009 pandemic H1N1, H3N2, or human B virus when their sera were tested by hemagglutination-inhibition (HI) assay with those viruses and 0.5% turkey red blood cells. All animal experiments were performed at the biosafety level 3 (BSL-3) facility approved by Korean government.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory animals of Korean veterinary quarantine and service. The protocol (2010-3-12) was approved by Committee on the Ethics of Animal Experiments of Chungnam National University.

Depletion of alveolar macrophages in the ferrets' lungs

We used liposome-encapsulated dichloromethylene diphosphonate (MDPCL2) (clodronate-liposome) (1.9–2.0 g/10 ml) that was cytotoxic to alveolar macrophages (Van Rooijen, 1989) to deplete alveolar macrophages in the lungs of ferrets *in vivo*. Phosphate buffered solution (PBS)-encapsulated liposome (PBS-liposome) was used as a control. The clodronate-liposome and PBS-liposome were obtained from Dr. Nico van Rooijen at Department of Cell Biology and Immunology, Free University, The Netherlands. Ferrets were intramuscularly (i.m.) anesthetized with Zoletil (2 mg/kg) (Virbac Laboratories, Carros, France) and intratracheally (i.t.) administered 0.5 ml of clodronate-liposome or PBS-liposome for 7 days (once per day) before ferrets were infected with 0.5 ml of 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus (A/California/07/2009), which was passaged 5 times in eggs. When we tried intranasal administration of clodronate-liposome to deplete alveolar macrophages in ferrets, we were not successful.

Determination of depletion efficiency of alveolar macrophages in the lungs of ferrets

We studied the depletion efficiency of alveolar macrophages with clodronate-liposome in the lungs of ferrets ($n=3$ per group) by counting cells in bronchoalveolar lavage (BAL) samples. Ferrets i.t. administered 0.5 ml of clodronate-liposome or PBS-liposome for 7 days were euthanized with a high doses of Zoletil, and the lungs with respiratory tracts (trachea and bronchus) were collected from the ferrets before they were lavaged with 50 ml of PBS (pH 7.4). The lavaged samples were centrifuged at 800*g* for 10 min to spin down cells and the collected cells were re-suspended with 5 ml of PBS (pH 7.4). One hundred microliter of the re-suspended cells was cytospined for Wright staining for differentiating cells. The cytospined cells were allowed to air dry on the slide glass and were fixed by immersing them in methanol for 5 min before Wright staining solution (Muto Pure Chemicals Co., Tokyo, Japan) was dropped on the top of cells on the glass slide. The slide was incubated for 2 min, and then Wright staining buffer was dropped on before 5-min incubation at room temperature. The glass slide was rinsed with the distilled water and then cells were counted under a light microscope.

Cell was differentiated by its morphology, nuclear morphology, and cytoplasmic granulation based on the counted 1000 cells in random fields of view under the light microscope.

Counting bacteria in BAL samples

The BAL samples from infected ferrets ($n=3$ per group) treated with clodronate-liposome and PBS-liposome at 5 days after infection were tested for bacterial infection. The serial 10-fold dilutions of BAL samples in PBS (pH 7.4) were transferred to a MacConkey agar plate, a tryptic soy agar plate, or blood agar. The number of bacterial colonies was counted at 72 h after incubation (37 °C).

Studying the clinical signs in the infected ferrets

Ferrets ($n=5$ per group) depleted of alveolar macrophages by clodronate-liposome or control ferrets administered with PBS-liposome were i.n. infected with 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus (A/California/07/2009). Respiratory signs and mortality in the infected ferrets were observed daily, and body weight and rectal temperature were measured for 14 days after infection.

Measurement of clinical scores of infected ferrets

Ferrets ($n=5$ per group) treated with clodronate-liposome or PBS-liposome were i.n. infected with 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus (A/California/07/2009). The clinical signs were observed for 14 days after infection. Clinical scores were the sum of activity and respiratory signs. The activity was graded as follows: 0 representing normal activity, 1 representing calm demeanor, and 2 representing depression and loss of interest in play. The respiratory signs were graded as follows: 0 representing the normal state, 1 representing occasional sneezing and serous nose exudates, and 2 representing frequent sneezing, coughing, and mucosal nose exudates.

Viral titration in the lungs of infected ferrets

One-gram parts of the left lung cranial lobes of ferrets ($n=3$ per group) infected with 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus (A/California/07/2009) at 3 and 5 days post inoculation (p.i.) were homogenized using homogenizer (Daihan Co., Seoul, Korea) and frozen and thawed three times in 1 ml of PBS (pH 7.4)

supplemented with 2× anti-biotic-antimycotic solution (Sigma, St. Louis, MO). The samples were serially 10-fold diluted in PBS (pH 7.4) and each diluted sample was inoculated into 10-day-old hens' eggs. The infection of virus in the inoculated eggs was determined by hemagglutination (HA) assay. Viral titers were determined by log 10 EID_{50}/ml as described (Reed and Muench, 1938). We selected the left cranial lobes of ferrets' lungs since the pathological legion is severer than other lobes to measure the viral titers, and collected tissues showing lesions on the left cranial lobes.

Histopathological staining

The ferrets ($n=3$ per group) infected with 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus (A/California/07/2009) were euthanized with high doses of Zoletil at 3 and 5 days p.i., and were thoroughly exsanguinated before the left cranial lobes of lungs were collected within biosafety cabinet (BSC). The lung tissues were fixed by submerging them in the 10% neutral buffered formalin and embedding in paraffin. Five-micrometer sections were carried out and stained with hematoxylin and eosin (H&E) as described (Bancroft and Stevens, 1996). The stained tissues were evaluated under an Olympus DP70 microscope (Olympus Corporation, Tokyo, Japan).

Table 1
Ferret's cytokine primers for quantitative real-time PCR.

cytokine	Forward primer	Reverse primer
IL-1α	ACCCACTTCATGAGGACTGC	TGCTACTGATCTGGGCTTCA
IL-1β	GGACTGCAAATTCCAGGACATAA	TTGGTTCACACTAGTTCCGTTGA
IL2	CTTCGCAAACAGTGCACCTA	GCCTTCTTGGGCATGTAGAA
IL-4	TCACCGGCACTTTCATCCA	TTCTCGCTGTGAGGATGTTCA
IL 5	GGGGAGGCTGTGGATAAACT	CAACTTTCCGGTGTCCACTC
IL-6	AGTGGCTGAAACACGTAACAATTC	ATGGCCCTCAGGCTGAACT
IL-8	AAGCAGGAAAACTGCCAAGAGA	GCCAGAAGAAACCTGACCAAAG
IL10	CCTGTCGGAGATGATCCAGT	CAAGCTCACTCATGGCTTTG
IL 11	CTGAGCCTGTGGCCAGATA	GGGAATCCAGGTTGTGGTC
IL12β	CACCAGCAGCTTCTTCATCA	AGGTCTTGTCCACGCAGAGT
IL 13	TGGTTGACTGTGGTCATTGC	GATGCCTGATTCTGGGTGAT
IL-16	GGGGAGCGGATCTAGAAAAC	CGAGGAGGAGTTCAGGTCAG
IL17	CCTCAGCATGTGAAGGTCAA	AACCAGGATCTCTTGCTGGA
IL 18	GAGGATATGCCCGATTCTGA	ATCATGGCCTGGAACACTTC
TNF-α	CCAGATGGCCTCCAACTAATCA	GGCTTGTCACTTGGAGTTCGA
IFN-γ	TCAAAGTGATGAATGATCTCTCACC	GCCGGGAAACACACTGTGAC

Immunohistochemistry staining

Ferrets ($n=3$ per group) uninfected or infected with 10^6 $TCID_{50}$ of pandemic H1N1 (A/California/07/2009) influenza virus were euthanized with high dose of Zoletil at 5 days after infection, and the left cranial lobes of lungs were collected. The lung tissues were fixed using 10% neutral-buffered formalin for 24 h and then embedded in paraffin. Five-micrometer sections were made and stained with mouse influenza A virus anti-nucleoprotein antibody (Serotech, United Kingdom). Tissue sections were deparaffinized and hydrated in distilled water. Sections were fixed with 100% chilled acetone for 2 h for permeabilization, and endogenous peroxidase activity was blocked by incubating tissue sections in 3% H_2O_2 for 15 min at 37 °C before the sections were blocked with 5% bovine serum albumin in PBS (pH 7.4) for 1 h. The blocked tissue sections were labeled with mouse influenza A virus anti-nucleoprotein antibody (1:1000 dilution) by incubating at room temperature for 1 h. The labeled tissue sections were stained with biotin-labeled goat anti-mouse immunoglobulin (Vector, USA), VECTASTAIN ABC-AP (Vector, USA), and Vector red alkaline phosphatase substrate (Vector, USA). The stained tissue sections were counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA) before the stained sections were evaluated under an Olympus DP70 microscope (Olympus Corporation, Tokyo, Japan).

Quantification of inflammatory cytokines and chemokines by quantitative real-time PCR

Total RNA was collected from lung tissues (1 g) of ferrets ($n=3$ per group) that were infected with 10^6 $TCID_{50}$ of 2009 pandemic H1N1 influenza virus using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the collected RNAs. SYBR Green-based real-time PCR was performed using a Roto-Gene 6000 apparatus (Corbett, Mortlake, Australia) and SensiMix Plus SYBR (Quantace, London, UK) based on the recommendations of the manufacturer. Samples were run in duplicate. A total volume of 20 μl containing 2 μl cDNA, 10 μl SYBR mixture, and inflammatory cytokine-specific primers for ferrets (Table 1) or chemokine-specific primers for ferrets (Table 2) (Kelvin et al., 2012) [1 μl of forward primer (20 pmole) and 1 μl of reverse primer (20 pmol)] was used for 40 cycles of PCR: 5 s at 95 °C, 15 s at 60 °C, and 25 s at 72 °C. Cytokine and toll-like receptor (TLR) expression levels in tissues were normalized to those of chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results of real-time PCR were quantified by the comparative threshold method after subtracting the data from uninfected control ferrets.

Table 2
Ferret's chemokine primers for quantitative real-time PCR.

Chemokine	Forward primer	Reverse primer
CCL2	GCTCCCTATTCACTTGCTGTTTC	GATTCGATAGCCCTCCAGCTT
CCL 4	TGTGACCGTCCTTTCTCTCC	GAATCTTCCGCAGGGTGTAA
CCL5	GCTGCTTTGCCTACATTTCC	CCCATTTCTTCTGTGGGTTG
CCL 7	TATCTCAACCACCTGCTGCT	GCTTGGGTTTTCTTGTCCAG
CCL8	CATCCCAATTACCTGCTGCT	ACTGGCTGTTGGTGATCCTC
CCL 11	AGGTCTCCGCAGCACTTCT	TATCCTTGGCCAGTTTGGTC
CCL 17	CGGGAGTGCTGCCTAGAGTA	CTTCACCCTCTTGTCCTTGG
CCL21	TCAGGCAGAGCTATGTGCAG	TCAGTCCTCTTGCAGCCTTT
CCL22	ACTGCACTCCTGGTTGTCCT	ATCTTCACCCAGGGCACTCT
CCL 23	ACGAATTCGATGTGCAAACA	AGCTGGCCCCTACTACCATT
CXCL5	GAGCTGCGTTGTGTGTGTTT	ACTTCCACCTTGGAGCACTG
CXCL9	GGTGGTGTTCCTCTTTTGTTGAGT	GGAACAGCGTCTATTCCTCATTG
CXCL10	CTTTGAACCAAAGTGCTGTTCTTATC	AGCGTGTAGTTCTAGAGAGAGGTACTC
CXCL 11	AGAGGACGCTGTCTTTGCAT	TGGGATTTAGGCATCGTTGT
CXCL12	ACAGATGTCCTTGCCGATTC	CCACTTCAATTTCGGGTCAA
CXCL 13	TCCAAGGTGTTCTGGAGGTC	GGGAATCTTTCTCTTAAACACTGG
CXCL 14	CCCTCCGGTCAGCATGAG	CCAGGCGTTGTACCACTTG

Statistical analysis

Statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package, version 10.0 (SPSS, Cary, NC, USA). The Student's *t*-test was used. A P-value < 0.05 was considered to be statistically significant.

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